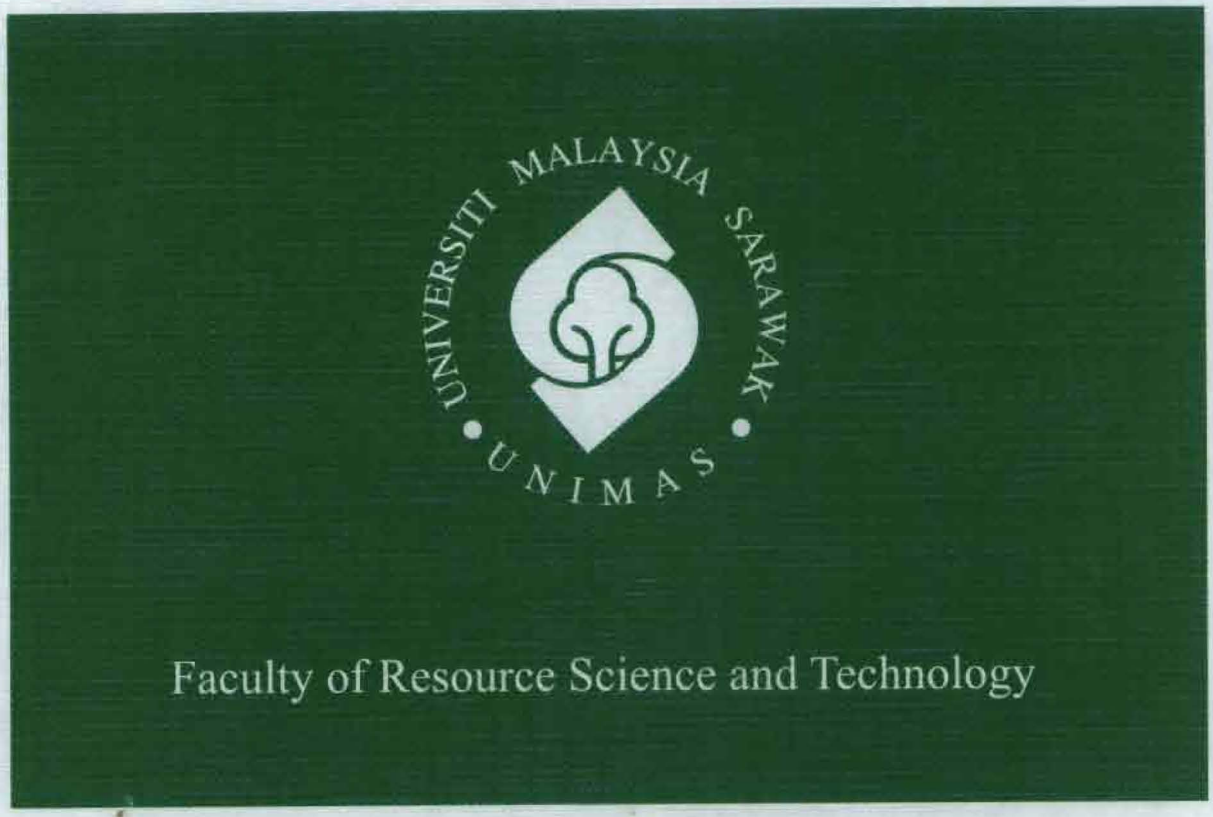

SIMULTANEOUS CO-SACCHARIFICATION AND FERMENTATION OF SAGO HAMPAS FOR BIOETHANOL PRODUCTION

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Bachelor of Science with Honours
(Resource Biotechnology)
2013

Simultaneous co-Saccharification and Fermentation of Sago Hampas for Bioethanol Production

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A final project report submitted in partial fulfilment of the
Final Year Project II (STF 3015) Resource Biotechnology

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2013

ACKNOWLEDGEMENT

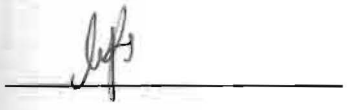
In the first place, I am thankful to God Almighty for His blessing and strength in completing this research. Special thanks to Dr. Micky Vincent and Ennry Esut for their supervision and constant support. Their valuable assistances and constructive comments throughout the experimental and thesis work have contributed to the success of this research.

My deepest appreciation is also extended to all the technicians at Faculty of Resource Science and Technology especially Mr. Azis Ajim and Mr. Leo Bungin for their co-operation. Also, a million thanks goes to the senior students in Microbiology Laboratory, Norizawati Muhamad Nur, Patricia Rowena Mark, and Yong Sy Fuh for their patient and guidance.

I am also equally grateful to my family for their endless love, prayer, encouragement and financial support. Special thanks to all my friends for their assistance and accompany in completing my research. All of them have been very helpful to me.

DECLARATION

I hereby declare that the thesis entitled "**Simultaneous co-Saccharification and Fermentation of Sago Hampas for Bioethanol Production**" is submitted for Resource Biotechnology degree at Universiti Malaysia Sarawak. It is my own work and has not been previously submitted anywhere for any award or any other university.



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LIST OF ABBREVIATIONS

ATCC	American Type Culture Collection
DNS	Dinitrosalicylic
FPU	Filter Paper Unit
g	gram
hr	hour
HPLC	High Performance Liquid Chromatography
IUPAC	International Union of Pure Applied Chemistry
LB	Luria Bertani
M	Molar
m	milli
ml	millilitre
NaOH	Sodium Hydroxide
NREL	National Renewable Energy Laboratory
rpm	Revolution per minute
Sc-SF	Simultaneous co-Saccharification and Fermentation
SHF	Separate Hydrolysis and Fermentation
SSF	Simultaneous Saccharification and Fermentation

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Simultaneous co-Saccharification and Fermentation of Sago Hampas For Bioethanol Production

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ABSTRACT

Abundance of lignocellulosic biomass provides a good solution to the demands of energy crops in producing biofuel like biodiesel and bioethanol. In this study, bioethanol was produced via the Simultaneous co-Saccharification and Fermentation (Sc-SF) process. The processing steps in Sc-SF were virtually the same as in Simultaneous Saccharification and Fermentation (SSF), except for the addition of two enzymes for saccharification. Amylase and cellulase were added for co-saccharification of sago starch residue and sago fibre. Then, *Saccharomyces cerevisiae* was used to ferment the hydrolysates. Samples were analysed using High Performance Liquid Chromatoghrphy (HPLC). Sc-SF showed high yields of hydrolysis and ethanol production within the first 6 hours of fermentation. Glucose production was 37.86 g/l for 5.0% sago hampas and 17.47 g/l for 2.5% sago hampas. The highest bioethanol yield in 5.0% sago hampas was 80.50%. Meanwhile, the highest bioethanol yield in 2.5% sago hampas was 73.19%. This study indicated that there is feasibility of increasing bioethanol production via Sc-SF.

Keywords: Bioethanol, sago hampas, Simultaneous co-Saccharification and Fermentation (Sc-SF), amylase, cellulase, *Saccharomyces cerevisiae, HPLC.*

ABSTRAK

Sumber biomas lignosellulosa yang banyak berpotensi untuk menyelesaikan perdebatan dalam penggunaan tanaman bagi menghasilkan biofuel seperti biodiesel dan bioethanol. Dalam kajian ini, bioetanol daripada hampas sagu dihasilkan melalui kaedah Sakarifikasi bersama Serentak dan Fermentasi (SbSF). Langkah pemprosesan hampas sagu dalam SbSF adalah hampir sama seperti Sakarifikasi Serentak dan Fermentasi (SSF). Namun demikian, terdapat dua enzim ditambah untuk proses sakarifikasi kanji dan bahan lignoselulosa yang merupakan komponen utama dalam hampas sagu. Enzim yang telah digunakan adalah amilase dan selulosa. Hidrolisat yang terbentuk digunakan oleh Saccharomyces cerevisiae *sebagai sumber tenaga lalu menghasilkan bioetanol melalui proses fermentasi. Sampel dianalisa menggunakan Kromatografi Cecair Berprestasi Tinggi (HPLC). Dalam 6 jam pertama fermentasi, SbSF menggunakan 2.5% dan 5.0% hampas sagu menunjukkan hasil hidrolisat dan bioetanol dalam jumlah yang tinggi. Pengeluaran glukosa yang tertinggi adalah 37.86 g/l untuk 5.0% hampas sagu dan 17.47 g/l untuk 2.5% hampas sagu. Penghasilan bioetanol tertinggi adalah 80.5% untuk 5.0% hampas sagu dan 73.19% untuk 2.5% hampas sagu. Kajian ini menunjukkan bahawa kaedah SbSF mampu meningkatkan penghasilan bioethanol.*

Kata kunci: Bioetanol, hampas sagu, Sakarifikasi bersama Serentak dengan Fermentasi (SbSF), amilase, selulosa, Saccharomyces cerevisiae, *HPLC.*

CHAPTER 1

INTRODUCTION

The demand on fossil fuels for energy has increased exponentially since the explosion of industries in the first world and developing countries (Sun and Cheng, 2002) and the increase is predicted to continue. On the other hand, global oil production is expected to decline from 25 billion barrels to 5 billion barrels by 2050 (Campbell and Laherree, 1998). Together with the continual fluctuation in oil prices, this phenomenon has sparked a renewed interest in the potential use of renewable sources such as lignocellulose to produce a variety of biofuels.

To date, the most important biofuels produced are biodiesel and bioethanol. Bioethanol is a liquid fuel produced from sources other than mineral reserves such as oil, coal and gas. The leading nations in bioethanol production are Brazil and USA (Carere *et al.*, 2008), whereas Asian countries altogether account for about 14% of world's bioethanol production.

Biofuel productions are basically characterized into two phases, first and second generation. First generation biofuels are produced primarily from food crops. In Brazil, about 70% of ethanol is produced from fresh sugar cane and the remaining percentage is from cane molasses (Wilkie *et al.*, 2000). Meanwhile, bioethanol in USA is produced almost exclusively from corn. The main concern regarding first generation biofuels is the impact biofuel production may have on land biodiversity and the competition with food crops (Pimental and Patzek, 2005; Mitchell, 2008). Thus, undefined long term viability of first generation process bioethanol has gather researcher's interest on the second generation processes.

Second generation biofuels are produced primarily from lignocellulosic biomass. These resources have widespread abundance. Lignocellulosic biomass comprises about 50% of the world biomass and its annual production was estimated in 10-50 billion ton (Claassen *et al.*, 1999). Besides, second generation biofuels is expected to provide both the short term benefits of first generation biofuels as well as provide long term benefits (Rajagopal *et al.*, 2007). Therefore, the production of bioethanol from lignocellulosic biomass is clearly one of the best energy alternatives. However, the main limiting factor is the higher degree of complexity inherent to the processing of lignocellulosic biomass.

The complexity of the biomass structure and properties produces lower concentration of glucose in enzymatic hydrolysis. Hence, in this study bioethanol was produced from ground sago hampas which was subjected to hydrolysis using amylase and cellulose. Then, the hydrolysates were fermented using the yeast, *Saccharomyces cerevisiae*.

The objectives of this study are to:

(i) Produce bioethanol by fermenting the hydrolysate through Simultaneous co-Saccharification and Fermentation (Sc-SF).

(ii) Increase the conversion rate of sago starch residue and sago fibre to fermentable sugar using amylase and cellulase.

(iii) Determine the feasibility of increasing bioethanol production via Simultaneous co-Saccharification and Fermentation (Sc-SF) of the fiber and residual starch in sago hampas.

CHAPTER 2

LITERATURE REVIEW

2.1 Ethanol and Bioethanol

Ethanol belongs to alcohol family and is commonly known as ethyl alcohol, grain alcohol, wine spirit, and cologne spirit. The molecular formula of ethanol is C_2H_5OH. In average, 73% of produced ethanol worldwide is fuel ethanol, 17% is beverage ethanol and the remaining 10% is industrial ethanol. In transportation, ethanol can be used as a gasoline enhancer instead of the common additive, methyl tertiary butyl ether (MTBE) (Wang *et al.*, 1999) or blended with gasoline for transportation fuels (Varga *et al.*, 2004; Ferreira *et al.*, 2010).

Early ethanol productions have always been via the anaerobic fermentation processes by yeast. The reaction is represented in the equation below:

$$C_6H_{12}O_6 \longrightarrow 2C_2H_5OH + 2CO_2$$

During early days, ethanol production was for beverages and lamp fuel. This ethanol is known as bioethanol as it was produced biologically via fermentation instead of mineral reserves such as oil, coal and gas. Currently, approximately 95% of bioethanol is produced biologically and approximately 5% is produced synthetically via hydration of ethane (Berg and Licht, 2004). The feedstock for ethanol production via fermentation is obtained from energy crops and lignocellulosic biomass (Gomez *et al.*, 2008).

2.2 Sago Hampas for Bioethanol Production

Sago palm belongs to the genus *Metroxylon* of the Palmae family. Habitats of sago palms are mainly tropical lowland forest and freshwater swamps. It is an extremely hardy plant as it immune to floods, drought, fire and strong winds. In addition, sago palm thrives in swampy, acidic peat soils, submerged and saline soils (Flach and Schuiling, 1989; Hasajima, 1994). Sago-palm derived products have many uses. For example, the leaves of sago are used in house construction and woven into bags, baskets, cages and ropes. Meanwhile, the piths are shredded and sedimented in water for starch extraction (Wina *et al.*, 1986). These activities create agro-residues.

Agro-residues can be defined as the parts of cultivated plants that are thrown out after industrial processes. There are three major types of agro-residues that were generated during the processing of sago starch. They are sago trunk, fibrous pith residue and wastewater (Figure 1).

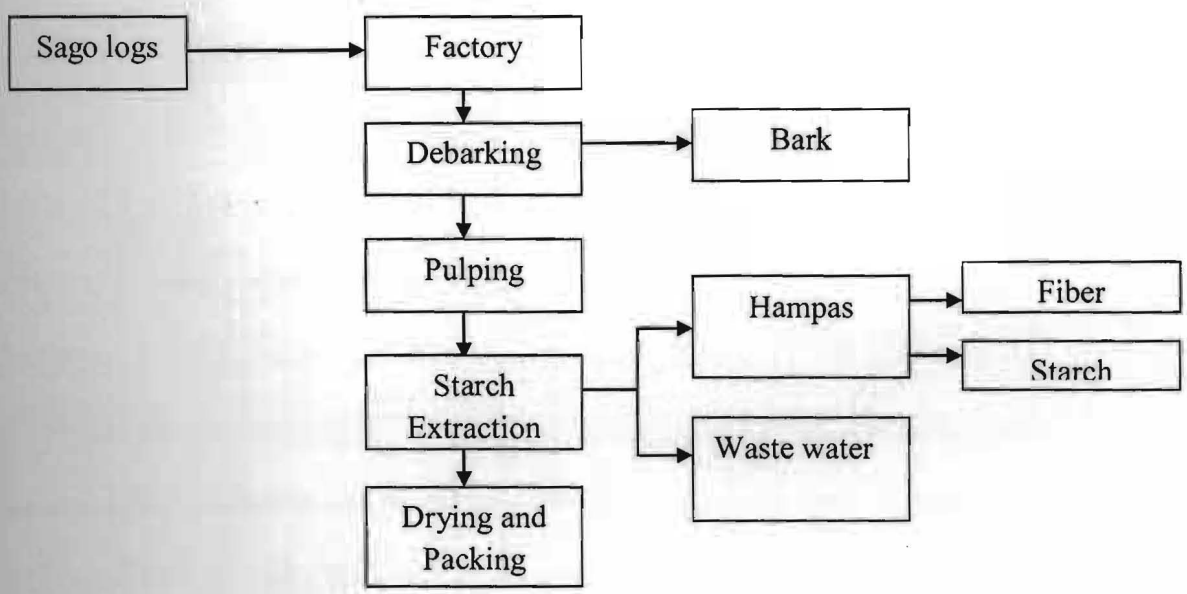

Figure 1: Schematic flow diagram for sago starch processing (Yean and Lan, 1993).

In Sarawak, agro-residues from sago starch processing industries are abundant and readily available. It is estimated that approximately 7 tons of sago piths waste are produced daily from a single sago starch processing mill (Bujang *et al.*, 1996). These residues are washed off into nearby streams together with wastewater and deposited in the factory's compound. Sago wastewater represents high organic material, chemical oxygen demand (COD) and biological oxygen demand (BOD). Instead of polluting the environment, sago hampas is better utilized for global environmental conservation and sustainable development through production of bioethanol via the second generation process.

2.3 Sago Hampas Composition and Structure

Sago hampas is a starchy lignocellulosic by-product generated from the pith of *Metroxylan sagu* after starch extraction. The amount of hampas released from sago processing factory depends on the quality of extraction process. According to Linggang *et al.* (2012), sago hampas contains approximately 58% starch, 23% cellulose, 9.2% hemicellulose, and 4% lignin on a dry weight basis. Moreover, according to Sun *et al.* (1999), the cellulose fraction consisted of 89% glucose and small amounts of other sugars such as xylose, rhamnose, arabinose, mannose, fructose and galactose.

Starch is a polysaccharide carbohydrate consisting of a large number of glucose units joined together by glycosidic bonds. It consist of amylose (14-27%) and amylopectin (73-86%) (Aehle, 2007). Amylose consist of approximately 200 to 6000 glucose units (MW 10^5-10^6) that are linked by α-1, 4 glucoside bonds to form linear chains (Figure 2).

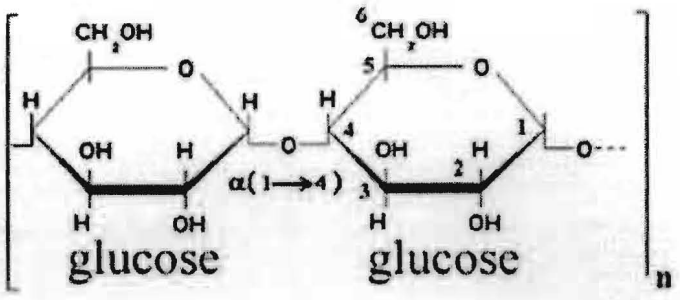

Figure 2: Chemical structure of amylose (Buleon, 1998)

On the other hand, amylopectin have a polymeric, branched structure (Figure 3) with up to $3x10^6$ glucose units and a MW of approximately $5x10^8$. It consists of connected branches that are linked by α-D-1, 6 glucoside bonds (≈95%) which occur at every 24 to 30 glucose units of the α-D-1, 4 glucoside bonds (5%) (Sun *et al.*, 2006).

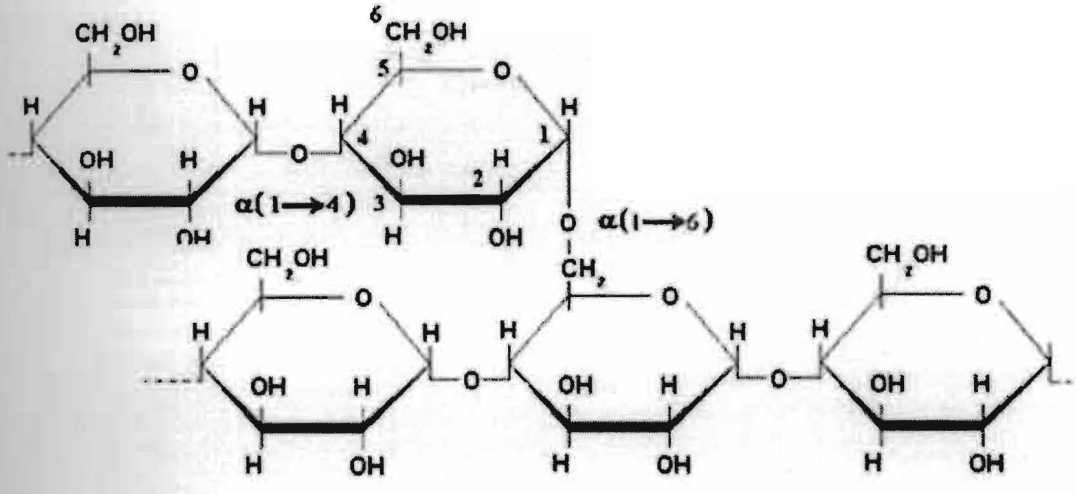

Figure 3: Chemical structure of amylopectin (Buleon, 1998)

Lignocellulosic material is made up of a matrix of cellulose and lignin bound by hemicelluloses chains. Cellulose is a homopolysaccharide composed of repeating β-D-glucopyranose units. It is enzymatically hydrolysable by three different cellulolytic enzymes: endoglucanases, exoglucanases and β-glucosidases (Vásquez *et al.*, 2007). Contrarily to cellulose, hemicellulose is heteropolysaccharide composed of glucose, galactose, arabinose,

and xylose (Mohamed and Duarteb, 2003). Hemicellulose is easily hydrolysable to fermentable sugars. Xylans and glucomannans are the most common compounds among them. Meanwhile, lignin is a complex polymer of phenyl propane which occurs primarily between the fibre cells. It acts as cementing agent which provide barrier to enzymatic attack (Howard *et al.*, 2003). Moreover, it is always associated with hemicellulase through carbon-carbon and ether linkages (Xu *et al.*, 2008).

2.4 Simultaneous co-Saccharification and Fermentation (Sc-SF)

The classic configuration employed for fermenting biomass hydrolysates involves a process where the hydrolysis of cellulose and fermentation are carried out in different units, known as Separate Hydrolysis and Fermentation (SHF). Simultaneous Saccharification and Fermentation (SSF) is an alternative variant to SHF. SSF combines enzymatic hydrolysis of cellulose with simultaneous fermentation of its main derived sugar to ethanol (Takagi *et al.*, 1997). Both hydrolysis and fermentation are performed in the same vessel unit.

SSF is more favorable as compared to SHF as SSF enabled the reduction of process steps number (Koon Ong, 2004), enzyme loading (Kadar *et al.*, 2003) and production cost (Ferreira *et al.*, 2010). Besides, SSF allowed rapid conversion of glucose into ethanol by yeast enabled the reduction of end product inhibition (Viikari *et al.*, 2007). Another advantage is ethanol toxicity which prevents unwanted microorganisms from contaminating the fermentation broth (Elumalai and Thangavelu, 2010) and hence maintained the optimum condition for *Saccharomyces cerevisiae* growth.

Simultaneous co-Saccharification and Fermentation (Sc-SF) is a modified version of Simultaneous Saccharification and Fermentation (SSF). In Sc-SF, amylose and cellulase are added at the same time for the hydrolysis of sago residue starch and sago fibre. The main role of enzymatic hydrolysis of starch is to effectively provide the conversion of two major starch polymer components (amylose and amylopectin) to fermentable sugar (Aggarwal *et al.*, 2001).

Enzymatic hydrolysis involves two processes which are liquefaction and saccharification. Liquefaction is the process of breaking the large particles of starch polymer. Saccharification is the stages of depolymerization which involves the formation of mono-, di- , and tri-

saccharides (Barsby *et al.*, 2003). The next step in bioethanol production is the addition of *S. cerevisiae*.

S. cerevisiae is a spherical or ellipsoidal unicellular fungus from the yeast family which is commonly known as brewer's yeast or baker's yeast. It reproduces asexually by budding or division (Bamforth, 2005) into a varied size, typically measuring from 3 to 8 μm in diameter. It is facultative anaerobe which requires a reduced carbon source to proliferate.

Many researchers stated that *S. cerevisiae* is the most suitable microorganism for ethanol production. It can grow on a variety of sugars with high substrate and ethanol tolerance (Chandel *et al.*, 2011). However, it is capable of metabolizing few types of sugar such as glucose, fructose and sucrose only (Rhee *et al.*, 1986; Nakamura *et al.*, 1997).

2.5 Reducing Sugar Assay

In dinitrosalicyclic (DNS) assay, the presence of free aldehyde group or ketone group in reducing sugar is the basis for their dosage quantification. This method was first mentioned by Summer and Sisler (1944) and modified by Miller (1959). In an alkaline medium, the reducing sugar like glucose reduces 3-5-dinitrosalicyclic acid to 3-amino-5-nitrosalicyclic acid, and formed orange color. Appropriate temperature control is needed for proper color development and color stability (Miller, 1959). The intensity of the orange color increased with the increase concentration of reducing sugar. However, sometimes color intensity is not the actual representation of reducing sugar concentrations as reducing sugar are degraded while the analysis is performed (Miller, 1959).

Besides dinitrosalicyclic acid, the DNS reagent contains phenol, sodium bisulphite, and alkaline buffer. The phenol optimizes the quantity of the color produced and sodium bisulphite stabilizes the color in the phenol presence (Miller, 1959). Sodium bisulphite also reacts with any oxygen present in the buffer. An alkaline buffer is added for the redox reaction between reducing sugar and DNS. DNS is added to stop the enzymatic reaction. Additionally, samples have to be boiled vigorously to promote full color development and absorbance of diluted samples can be read at 540 nm (Zhang *et al.*, 2009).

2.5 High Performance Liquid Chromatography (HPLC)

High Performance Liquid Chromatography (HPLC) is a separation technique of carbohydrate to obtain information regarding a mixture and its component. The mixtures are separated on the basis of their polarity (Angelika *et al.*, 2001) between two phases in the chromatographic column (Meyer, 2010) to be identified and quantified by detectors and data handling systems. One phase is stationary and the other is mobile phase. The stationary phase is either a solid, porous, surface active material in a small particle form or a coated thin film while the mobile phase is a liquid. Meanwhile, mobile phase used is 5 mM H_2SO_4 at a flow rate of 0.8 ml/min and a 20 µl injection volume (Zacchi *et al.*, 2000; Karimi *et al.*, 2006; Vincent *et al.*, 2011).A typical HPLC system consists of a separation column, guard column, a thermostated autosampler, a quaternary pump and a refractive index detector (Figure 4).

Figure 4: High Performance Liquid Chromatography (Shimadzu/LC-20A, Tokyo, Japan) in FRST UNIMAS.

CHAPTER 3

MATERIALS AND METHODS

3.1 Materials

The materials used in this study:

1. Sago "hampas"
2. Cellulase enzyme (Accelerase 1500, Genencore, United States)
3. Amylase enzyme (Dextrozyme, NOVOZYME, Denmark)
4. *Saccharomyces cerevisiae* (ATCC 24859)
5. 10X YP Solution
    - 10 g yeast
    - 20 g peptone
6. DNS Reagent
    - 1 g dinitrosalicylic acid
    - 200 mg crystalline phenol
    - 50 mg sodium sulphite
    - 100 ml 1% NaOH
7. 1 M Citrate buffer
    - 210 g Citric acid monohydrate
    - 750 ml Deionised water
    - 50 to 60 g Sodium hydroxide, pH 4.3
    - 1416 ml Distilled water

3.2 Sample Collection and Preparations

Sago hampas was obtained from Pusa, Sarawak. The hampas was packed into porous plastic bags and left to stand for 1-2 days. This was done to allow water from the wet hampas to drain off naturally. Then, dried sago hampas was grinded to pass a 1 mm screen.

3.3 Enzymes

The commercial saccharification enzymes used in this study was Dextrozyme (5.56 U/ml) and Accelerase 1500 (50 FPU/g cellulose). Accelerase is a cellulases complex produced from genetically modified strains of *Trichoderma reesei*. This enzyme preparation contained multiple enzyme activities, mainly exoglucanase, endoglucanase, hemi-cellulase and beta-glucosidase. The concentration of each enzymes mixture added to fermentation broth is stated in Table 1.

Table1: Different percentage of sago hampas in each batch of Sc-SF and corresponding amount of enzymes added

150 ml	Hampas	Starch (58%)	Dextrozyme (5 μ per g)	Cellulose (23%)	Accelerase (50 FPU per g)	50 mM CB-YP
2.5%	3.75 g	2.175	10.875	0.8625	0.25 ml	145.25 ml
5.0 %	7.50 g	4.350	21.750	1.7250	1.50 ml	141.00 ml

3.4 Preparation of Fermentative Microorganisms

Saccharomyces cerevisiae was obtained from the ATCC collection. The *S. cerevisiae* culture was prepared by growing 1 vial of cultures overnight in 100 ml of sterile LB broth at 32 °C with constant agitation at 120 rpm. The *S. cerevisiae* cells were then harvested via centrifugation in two 50 ml conical centrifuge tubes for 5 minutes at 6000 rpm. Figure 5 shows harvested *S. cerevisiae* for fermentation and turbidity changes in Luria Bertani broth innoculated with *S. cerevisiae* at 0 hr and 24 hrs.

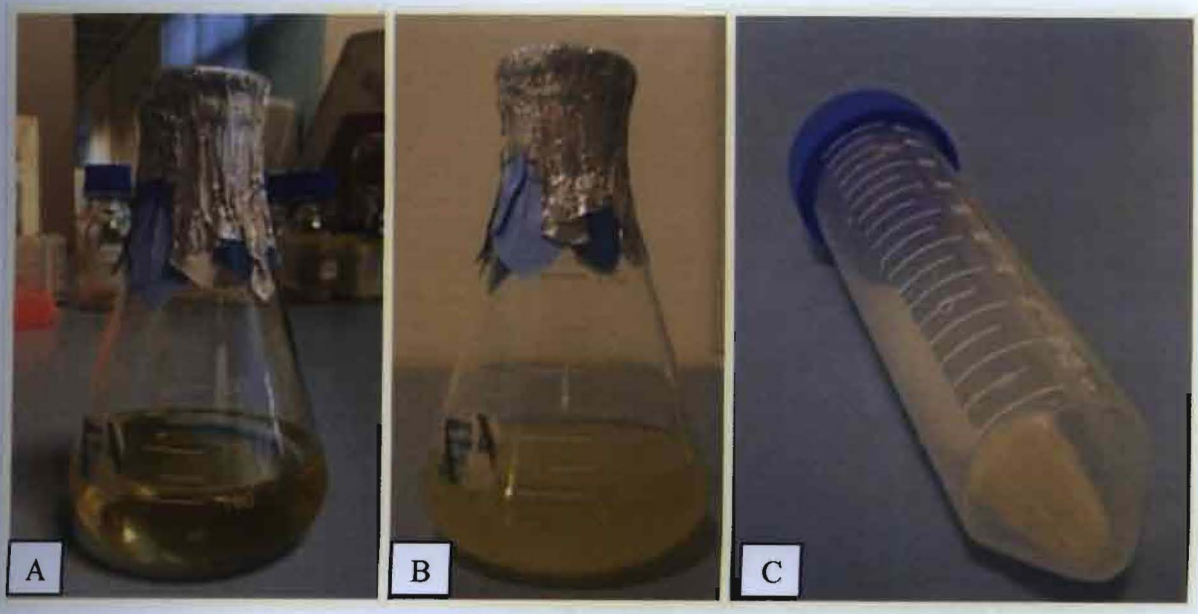

Figure 5: *S. cerevisiae* cultured in 100 ml LB broth at 0 hr (A) and 24 hrs (B) before harvested (C) for fermentation.